Ultra-High Carbon Dioxide Levels Enhances *in vitro* Shoot Growth and Morphogenesis in Labiatae

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ABSTRACT. The growth and morphogenetic development in shoot cultures of lemon basil (*Ocimum basilicum*), oregano (*Origanum vulgare*), peppermint (*Mentha* × *piperita*), spearmint (*Mentha spicata*), and thyme (*Thymus vulgaris*) shoots on MS salts and 0.8 percent agar containing 0 or 3 percent sucrose were determined under 350, 1,500, 3,000, 10,000, and 30,000 µl CO_2/liter of air were determined after 8 weeks growth. High CO_2 levels, especially the ultra-high levels (≥ 3,000 µl CO_2/liter of air), substantially increased fresh weight and leaf and root numbers in all cultures, whether or not the basal media contained sucrose, as compared with cultures grown on the same basal media under ambient air (350 µl CO_2/liter of air). Ultra-high CO_2 levels, also, enhanced the formation of aerial adventitious roots from the shoots of all species tested. A photosynthetic photon flux density of 180 µmole · $m^{-2} \cdot s^{-1}$ using a 16 h light/8 h dark photoperiod consistently provided better growth than lower light levels. *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-342-9678. E-mail address: <getinfo@haworthpressinc.com> Website: <http://www.haworthpressinc.com>]*

KEYWORDS. Adventitious rooting, lemon basil, oregano, peppermint, rooting, shooting, spearmint, thyme, tissue culture

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INTRODUCTION

Stimulation of *in vitro* photosynthesis in plants by CO_2 enrichment of the environment has been reported by many researchers with numerous species (1,2,3,4,6,13,16). Such elevation of CO_2 may enhance biomass production (2,3,12,13,14), rooting (5,9,14,18), leaf production (5,11,14), leaf area production (5), and shoot formation (5,14,18). In addition, biochemical production (4) and net photosynthesis (4,9) improve in cultures grown under elevated CO_2 levels. The elevation of CO_2 levels can be achieved by either forced ventilation, the direct pumping of gas mixtures into culture vessels (3), or by passive diffusion, the elevation of CO_2 levels within enclosed rooms or chambers containing culture vessels (2,3,12,13,17,18). Passive diffusion is the most common type of CO_2 addition.

Buddendorf-Joosten and Woltering (2) suggested that the optimum CO_2 concentration to achieve greatest growth rates varies among species. Only a limited range of CO_2 levels, however, have been tested on various species in elevated CO_2 environments and elevated CO_2 levels are often employed as one of many environmental variables studied concurrently (3,4,12,13,16). The use of CO_2 in tissue culture studies needs to be quantified similar to other growth factors to determine if the use of increased levels of CO_2 will result in increased growth and morphogenesis.

This study investigated the influence of various CO_2 levels on the culture of several members of the Labiatae family.

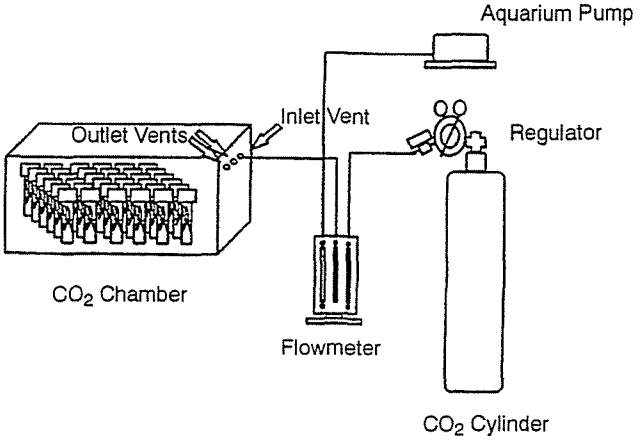
MATERIALS AND METHODS

Plant material. Shoots of lemon basil (*Ocimum basilicum* L.), oregano (*Origanum vulgare* L.), peppermint (*Mentha* × *piperita* L.), spearmint (*Mentha spicata* L.), and thyme (*Thymus vulgaris* L.) sustained on basal medium containing three percent sucrose under ambient air prior to testing were used as stock plant material in this study. Stock shoots were maintained in a culture room maintained at 25°C ± 1°C. Light in the culture room was supplied by a combination of cool white fluorescent tubes, metal-halide and incandescent lamps at a total photosynthetic photon flux density (PPFD) of 180 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the periphery of the basal media containers in a 16 h light and 8 h dark cycle.

The basal medium consisted of Murashige and Skoog (15) salts plus 0.5 mg thiamine-HCl, 100 mg myo-inositol, 10 g agar (Difco Laboratories, Detroit, MI)/liter, and 0 or 3 percent sucrose. The medium was adjusted to pH 5.7 ± 0.1 with 0.1 N HCl or NaOH before the addition of the agar. Subsequently, the agar was melted and then dispensed in 25-ml aliquots into 25 mm × 150 mm borosilicate glass culture tubes. The tubes with medium were capped with translucent polypropylene closures (Sigma Chemical CO., St. Louis, MO) and autoclaved for 15 min at 1.05 kg/cm^2 at 121°C. Tubes were placed at a 45 degree angle during cooling of the medium.

CO_2 treatment chambers. Plant cultures were treated in testing chambers, consisting of a transparent polycarbonate box and lid (Consolidated Plastics, Twinsburg, OH) (32.5 cm × 30 cm × 26.3 cm; W × L × D, 17.6-liter capacity) with three polypropylene spigots (Ark-Plas Products, Flippin, AR) attached to 0.45 µm air vents (Gelman Science, Ann Arbor, MI) (Figure 1). A silicone tape gasket (Furon, New Haven, CT) was attached to the lid and the chamber and lid were clamped together with 10 equally spaced stationary binding clips (30 mm × 50 mm × 25 mm; W × L × H). CO_2 (99.8% BOC Gases, Edison, NJ) from a gas cylinder, was mixed with ambient air (350 µl/liter CO_2) from an aquarium air pump (Whisper 2000, Carolina

FIGURE 1. Diagram of CO_2 enrichment system.



Mixing of the CO_2 and air generated by the pump occurred in the flow meter.

Biological Supply Company, Burlington, NC) for flowing through the test chambers. The flow mixture was controlled via a meter (Cole Parmer Instrument Co., Niles, IL) to provide 350 µl CO_2 (ambient air)/liter of air (control) and elevated levels of CO_2 to the chambers. CO_2 levels $\geq$ 10,000 µl/liter were monitored using a LIRA infrared gas analyzer (model # 3000, Mine Safety Appliances Company, Pittsburgh, PA) and CO_2 levels $\leq$ 3,000 µl/liter were monitored with a Li-Cor CO_2/H_2O infrared gas analyzer (model LI-6262, Li-Cor, Inc., Lincoln, NE).

The CO_2-air flows into the test chamber was 2000 ml/min during the light cycle. Gas exchange in the culture tubes was 1.4 exchanges/h (18) and experimental CO_2 concentrations within culture tubes were reached within 45 min following initiation of gas exchange and maintained for the duration for the photoperiod. The ultra-high CO_2 levels used in this study (3000, 10,000, and 30,000 µl CO_2/liter air) within culture tubes were measured using calibrated CO_2 electrode probes (model 501 mini, Diamond-General Dev. Corp., Ann Arbor, MI) placed in tubes and in the chamber (without plants and with thyme plants). Neither CO_2 nor air was flowed through the chambers during the dark cycle.

Experimental. For exposure to elevated CO_2, a single 2-cm long shoot of each species, taken from the stock plants, was cultured on 25 ml of fresh basal media (with 0 or 3% sucrose) in culture tubes. The culture tubes containing the experimental plant shoots were placed in the CO_2 treatment chambers in the previously described culture room (except for those samples in natural sunlight) and the selected CO_2 levels established. Preliminary experiments using CO_2 levels of 350 (ambient air), 1,500, 3,000, 10,000 and 30,000 µl CO_2/liter of air demonstrated the optimum CO_2 level was 10,000 µl CO_2/liter of air for the set of experimental plants.

To determine the effect of ultra high CO_2 levels on growth, sets of experimental plant shoot cultures on BM with and without sucrose were exposed to 350 (ambient air), 10,000, 30,000, and 50,000 µl CO_2/liter of air under a 16 h light-8 h dark cycle. The influence of light intensity on growth of peppermint and thyme shoots cultured on BM with and without sucrose was studied utilizing the ambient and the optimum CO_2 levels and light regimes (using the combination of lights in the culture room) of 80 and 180 $\mu mole \cdot m^{-2} \cdot s^{-1}$ PFFD under a 16 h light-8 h dark cycle and a light regime of $\leq$1000

$\mu mole \cdot m^{-2} \cdot s^{-1}$ provided by natural sunlight within a greenhouse using the seasonal photoperiod (spring and summer months of 1997).

After eight weeks of incubation, culture fresh weight, leaf numbers, root numbers, and shoot numbers were recorded for each experiment and analyzed with Student-Newman-Keuls multiple range test as appropriate. All experiments were repeated three times employing 10 to 20 replications/treatment.

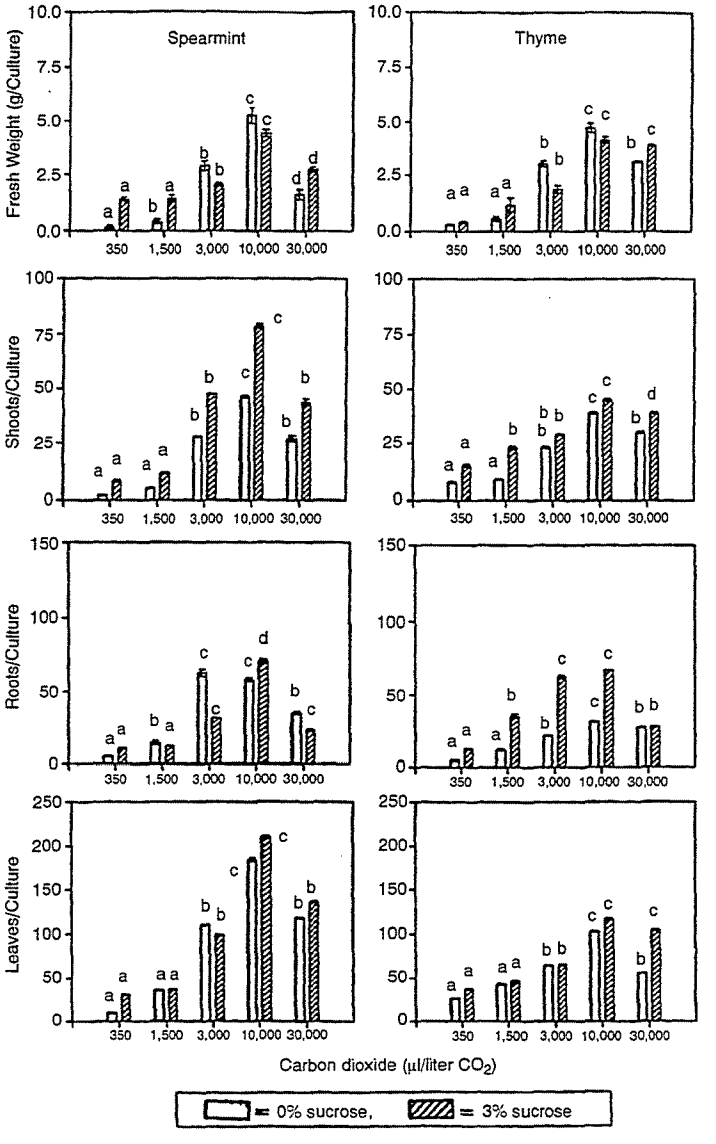
RESULTS

Significant increases in culture fresh weight, axillary shooting, leaf production, and rooting occurred in spearmint and thyme shoots on BM containing zero or three percent sucrose under levels of $CO_2 \geq 3{,}000$ µl CO_2/liter of air as compared with similar shoots under ambient air (Figures 2, 3, and 4). Similar results were obtained for lemon basil, oregano and peppermint shoots (Figure 5), except these species exhibited considerably lower rates of shoot production than shoots of spearmint or thyme. Invariably, shoot cultures grown under ambient air containing 350 µl CO_2/liter of air produced the lowest growth (fresh weight) and fewest morphogenic (leaf, root, and shoot numbers) responses. The highest growth and most morphogenic responses were invariably obtained in the experimental shoots grown at 10,000 µl CO_2/liter of air.

The photomixotrophic condition (BM with 3% sucrose under 10,000 µl CO_2/liter of air) usually produced the same results as exhibited in the cultures grown in the photoautotrophic condition (BM with 0% sucrose at 10,000 µl CO_2/liter of air). Both of the photomixotrophic and the photoautotrophic treatments produced growth responses superior to those of the traditional heterotrophic condition (BM with 3% sucrose with ambient CO_2 levels). No beneficial effect, as compared with 10,000 µl CO_2/liter of air, was obtained by using 30,000 or 50,000 µl CO_2/liter of air. Slightly lower growth and morphogenic responses consistently occurred at CO_2 levels above 10,000 µl CO_2/liter of air.

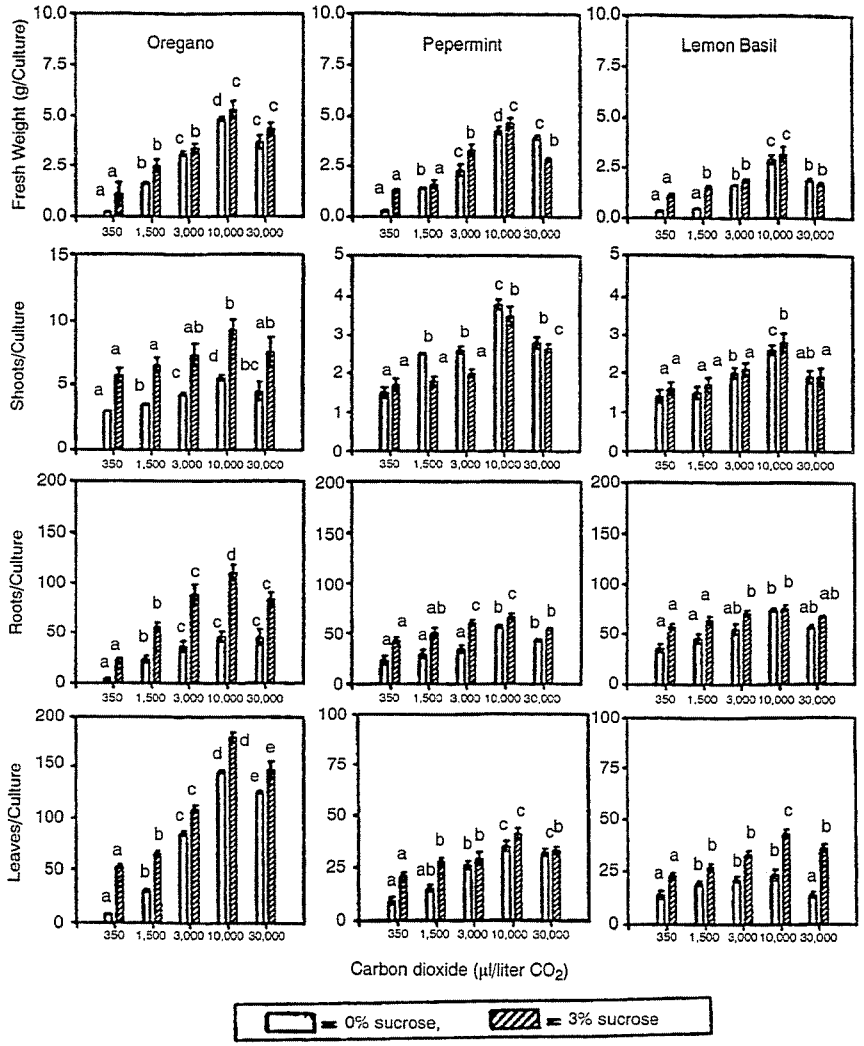
The most growth (fresh weight) of peppermint and thyme cultures consistently occurred under the 180 PPFD as compared with the 80 PPFD or the natural light conditions ($\leq$ 1,000 PPFD) at the ambient and optimum CO_2 levels (Figure 6).

FIGURE 2. Growth and morphogenesis in spearmint and thyme shoots.



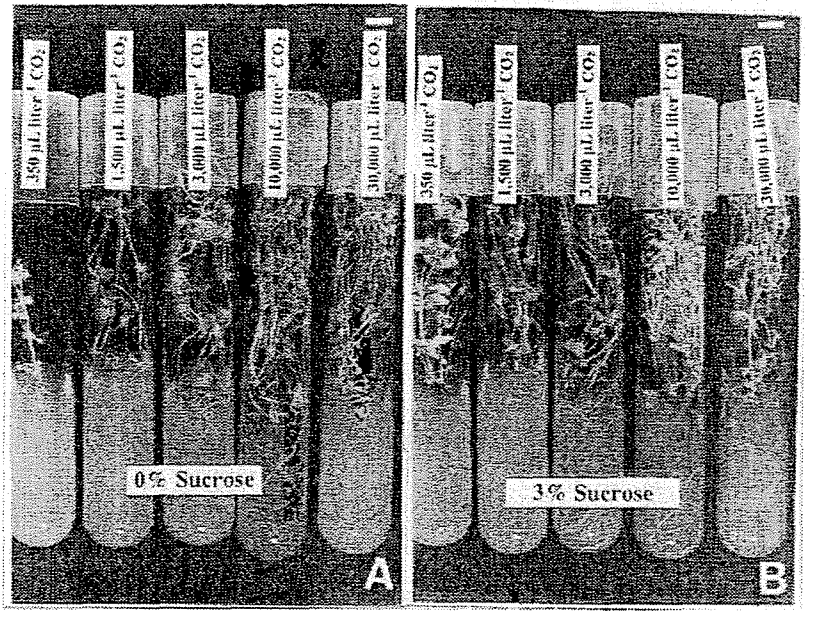
Shoots were cultured 1 per tube; means of 3 replicates, 10 samples per replicate, were separated Student-Newman-Keuls multiple range test ($P < 0.01$). Columns within the same sucrose level having the same letter were not significantly different.

FIGURE 3. Growth and morphogenesis in oregano, peppermint, and thyme shoots.



Shoots were cultured 1 per tube; means of 3 replicates, 10 samples per replicate, were separated Student-Newman-Keuls multiple range test ($P < 0.1$). Columns within the same sucrose level having the same letter were not significantly different.

FIGURE 4. Spearmint shoot cultures in enhanced CO_2 levels.

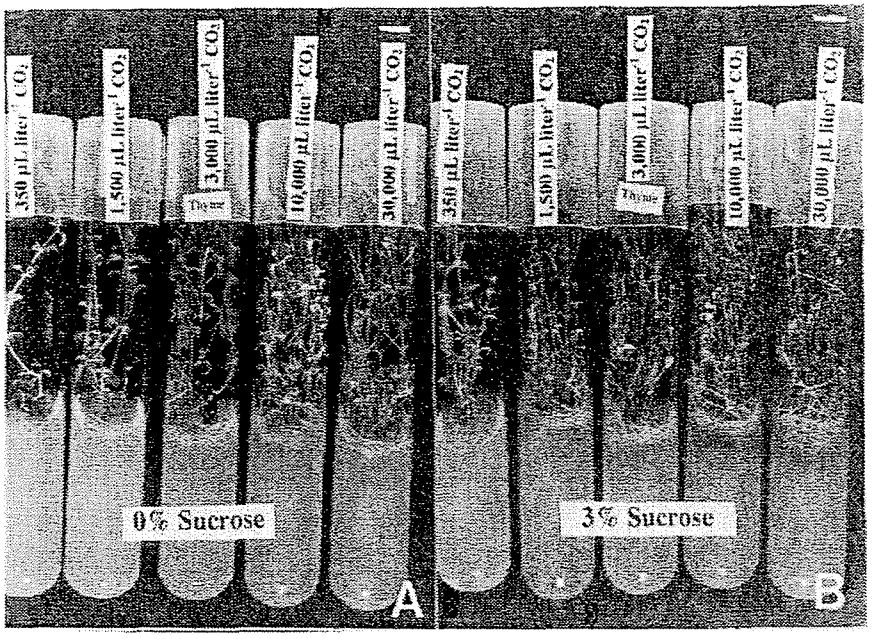


Photograph at 8 weeks in culture; note occurrence of aerial adventitious roots in the higher CO_2 treatments; bar = 10 mm.

DISCUSSION

Cultures of various species in the Labiatae family grown under ultra-high carbon dioxide levels appear to be able to utilize these carbon dioxide levels for enhanced photosynthesis that is subsequently translated into enhanced biomass (fresh weight) and morphogenic responses (leaves, roots, and shoots). These observations suggest carbon dioxide is a limiting nutrient *in vitro* that given in abundance to species of Labiatae results in enhanced growth and morphogenesis. No sucrose-CO_2 synergistic effect was noted in this study for Labiatae species using ultra-high CO_2 levels despite some suggestion that this combination provides an advantage for potato growth (6,7). In addition, leaf, root and shoot numbers from shoots of the Labiatae species studied were highest when cultured at 10,000 µl CO_2/liter of air whether sucrose was employed or not.

FIGURE 5. Thyme shoot cultures in enhanced CO_2 levels.

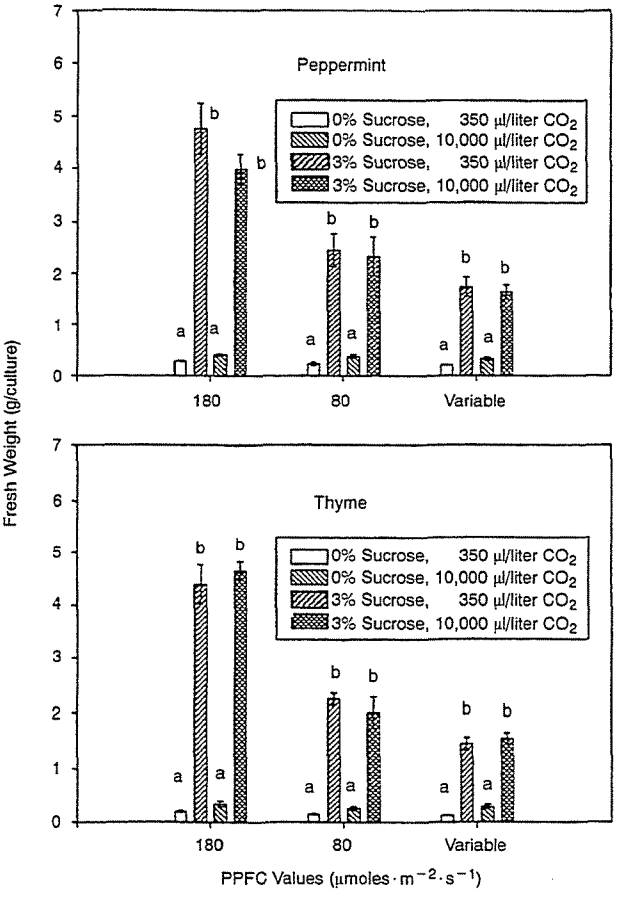


Photograph at 8 weeks in culture; note occurrence of aerial adventitious roots in the higher CO_2 treatments; bar = 10 mm.

The *in vitro* shooting capacity among the tested Labiatae species varied considerably. Spearmint and thyme could be considered "high" shoot producing species while oregano, peppermint, and lemon basil are "low" shoot producing species *in vitro*. Nevertheless, treatment with ultra-high levels of carbon dioxide enhanced shooting regardless of any inherent shooting ability among the tested species. The high occurrence of aerial adventitious roots observed on plants in our studies can most likely be attributed to the high relative humidity (100%) in the growth environment coupled with excessive photosynthetic products generated by leaves in cultures grown in the ultra-high CO_2 environment. The beneficial effect of CO_2 on rooting that has been mentioned in other plants *in vitro* (5,9,14,18).

The influence of carbon dioxide on *in vitro* production has been infrequently addressed (5,14,18). Most reports dealing with enhanced morphogenesis pertain to root and leaf growth and shoot lengths (18).

FIGURE 6. Growth of peppermint and thyme shoot cultures exposed elevated CO_2 and supplemental light.



PPFD of 180 and 80 µmole·m^{-2}·s^{-1} were provided from an artificial light source and the variable light level were provided by sunlight under greenhouse conditions; experiments were repeated 3 times with a single representation presented; mean of 10 samples separated by Student-Newman-Keuls multiple range test ($P < 0.1$).

In this study, we observed that carbon dioxide has an obvious beneficial effect on shoot production, an important morphogenesis process to plant propagators. Buddendorf-Joosten and Woltering (2) noted that leaf number and leaf area were greater in potato shoots grown under 50,000 µl CO_2/liter of air as compared with cultures grown under

ambient air. In contrast, Cournac et al. (4) observed no significant difference in the number of potato plantlet leaves grown under ambient air or 20,000 µl of CO_2/liter of air. Tisserat et al. (18) have reported that leaf number in lettuce grown under the ultra-high CO_2 levels increased significantly over that of cultures grown in ambient air.

Our results confirm previous finding (2,18) indicating ultra-high levels of CO_2, especially 10,000 µl CO_2/liter of air, will enhance leaf production of several Labiatae species and confirms the beneficial effects of CO_2 on rooting in other plants (5,9,14,18). Figueira et al. (5) observed that 20,000 µl of CO_2/liter of air induced cacao shoots on medium containing auxins to produce more leaves and roots than cultures grown under ambient air. Mitra et al. (14) enhanced rooting of *Chrysanthemum* shoots on medium containing auxins and sucrose with 20,000 µl CO_2/liter of air as compared with cultures grown under ambient air. We observed that plantlets grown without hormones in the medium appear more "normal" than similar plantlets grown on medium containing either auxins or cytokinins. Furthermore, we observed that the auxins and cytokinins produced undesirable callusing and plantlet and organ bloating.

Increases in photosynthetic rate under increases in CO_2 concentration and PPFD values occurs until a saturation point is reached (500 to 1,000 µl CO_2/liter of air with 100 PPFD for shade-grown leaves and 2000 PPFD for sun-grown leaves) (10). Our study confirm that increasing the PPFD values with elevated CO_2 levels increases growth (4,17). In a tissue culture environment used in this study, 180 PPFD may be considered "high," while 80 PPFD may be considered more "common." Clearly, the "common" PPFD values in our study did not provide enough light to achieve the high growth rates using ultra-high CO_2 levels obtained under "high" PPFD values. The relatively poor growth obtained in cultures in the greenhouse as compared with culture growth under artificial illumination in this study are probably due to daily variation in the PPFD within the greenhouse.

Although comparison of our results with other studies is difficult due to differences in such variables as rates of CO_2 application, CO_2 levels, system features, and culture vessel types, some similarities and divergence in plant response were apparent. In our study, the ultra-high levels of CO_2 ($\geq$ 3,000 µl CO_2/liter of air) remained relatively constant during daytime culture, but Figueira et al. (5) noted that CO_2 levels in cultures of cacao plants with ambient air dropped dramatical-

ly during the day and CO_2 levels of similar cultures with 20,000 µl CO_2/liter of air remained constant. These results suggest that ultra-high levels can provide sufficient CO_2 to compensate for lower levels in culture vessels, while ambient air with lower CO_2 levels fails to provide adequate CO_2 for photosynthesis. Kozai and Iwanami (12) observed that 30 day old carnation cultures grown in vessels in 1,500 µl CO_2/liter of air contained only 420 µl CO_2/liter of air (about 30% of that outside the vessel), demonstrating that high levels of CO_2 levels (1,500 µl CO_2/liter of air) could not be maintained within culture vessels under high photosynthetic rates and thus, possibly explaining the poor growth consistently observed at these CO_2 levels in our studies versus continued growth at the ultra-high CO_2 levels ($\geq$ 3,000 µl CO_2/liter of air). Lower CO_2 levels inside culture vessels than outside culture vessels were also observed by Kozai et al. (13). Statements by Jeong et al. (8), suggesting that optimum CO_2 levels for plant tissue cultures are 500 to 800 µl CO_2/liter of air, and Kozai (10), suggesting these CO_2 levels could be achieved within the culture tube by raising the CO_2 levels outside the tube to 5,000 µl CO_2/liter of air, cannot be supported by the available data.

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